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Docket Number:	82402-3693

PROVISIONAL APPLICATION FOR PATENT COVER SHEET (Small Entity)

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53 (c).

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TITLE OF THE INVENTION (280 characters max)

USE OF PLANT HEMOGLOBINS TO MAINTAIN CELL ENERGY STATUS

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☒	Specification	*Number of Pages*	25	☐	Small Entity Statement
☒	Drawing(s)	*Number of Sheets*	12	☐	Other (specify)

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT *(check one)*

		FILING FEE AMOUNT
☐	A check or money order is enclosed to cover the filing fees	
☐	The Commissioner is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number: [ ]	$75.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☒ No.

☐ Yes, the name of the U.S. Government agency and the Government contract number are: _____

Respectfully submitted,

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USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

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P19SMALLREV04

PROVISIONAL APPLICATION FOR PATENT COVER SHEET (Small Entity)

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Certificate of Mailing by Express Mail

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THE PATENT OFFICE HEREBY ACKNOWLEDGES RECEIPT OF THE FOLLOWING

DATED: Jun 26, 1998

Filing: Provisional Cover Sheet; Transmittal Letter filing the application no fees, no forms; Specification 25 pages; drawings 12 sheets;

APPLICANT: Aleksander W. Sowa

SERIAL NO.

CASE NO. 82402-3693

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June 26, 1998

PATENT APPLICATION
Commissioner of Patents & Trademarks
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Dear Sir:

RE: United States Patent Application
Aleksander W. Sowa, Phillip A. Guy, Stephen M. G. Duff, Xianzhou Nie and Douglas C. Dumin, Robert D. Hill

Being duly authorized and appointed, the undersigned is filing the attached provisional application pursuant to the provisions of revised 37 CFR 1.41(c) and 1.53(d) on behalf of the inventor, whose full name and address are as follows:

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ATTENTION: MICHAEL R. WILLIAMS

We attach a Declaration providing relevant details of this application. However this Declaration is NOT SIGNED. This application is being filed without a filing fee.

An appropriate signed Declaration and the filing fee will be filed upon receipt of The Notice of Missing Parts.

Granting of a filing date is respectfully solicited.

Respectfully submitted,

MURRAY E. THRIFT
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Enc

USE OF PLANT HEMOGLOBINS TO MAINTAIN CELL ENERGY STATUS

The present invention relates generally to the field of transgenic plants and expression vectors.

BACKGROUND OF THE INVENTION

Hemoglobins are widespread throughout the biosphere (Wittenberg and Wittenberg, 1990, *Annu Rev Biophys Biophys Chem* **19**:217-241). They are found in a broad range of organisms from bacteria, through unicellular eukaryotes, to plants and animals, suggesting that they predate divergence of life into plant and animal forms. Plant hemoglobins have been classified into symbiotic and nonsymbiotic types (Appleby, 1992, *Sci Progress* **76**:365-398): symbiotic hemoglobins are found in plants that are capable of participating in microbial symbioses, where they function in regulating oxygen supply to nitrogen fixing bacteria; nonsymbiotic hemoglobins have only recently been discovered and are thought to be the evolutionary predecessors of the more specialized symbiotic leghemoglobins. The ubiquitous nature of nonsymbiotic hemoglobins is evidenced by their broad presence across the plant kingdom (Appleby, 1985, Nitrogen Fixation and CO_2 Metabolism, eds. Ludden and Burris, pp. 41-51). The widespread presence and long evolutionary history of plant hemoglobins suggest a major role for them in the life of plants. Very little, however, is known about their function, although it has been proposed that nonsymbiotic hemoglobins may act either as oxygen carriers to facilitate oxygen diffusion, or oxygen sensors to regulate expression of anaerobic proteins during periods of low oxygen supply. The proteins from barley (Duff et al, 1997, *J Biol Chem* **272**:16746-

16752) and rice (Arredono-Pet r et al, 1997, *Plant Physiol* 115:1259-1266) and AHB1 from *Arabidopsis* (Trevaskis et al, 1997, *Proc Natl Acad Sci* 94:12230-12234) have been shown to have high oxygen avidity, with dissociation constants for oxyhemoglobin of 2.86 nM, 0.55 nM and 1.6 nM respectively, resulting in conditions whereby the free protein will remain oxygenated at oxygen concentrations far below those at which anaerobic processes are activated. Thus, it is unlikely that these hemoglobins would function as either facilitators of oxygen diffusion or sensors of oxygen, unless the oxygen avidity was modified by interaction with another component within the cell. Herein, it is shown that nonsymbiotic hemoglobins function to maintain the energy status of cells exposed to low oxygen tensions and that this property may be a common feature of plant cells, either during exposure to hypoxia or under high energy demand.

SUMMARY OF THE INVENTION

According to one aspect of the invention there is provided a recombinant expression system capable, when transformed into a plant, of expressing a gene encoding a nonsymbiotic hemoglobin, which system comprises a nucleotide sequence encoding said nonsymbiotic hemoglobin operably linked to control sequences effective in said plant.

The control sequences may include a strong constitutive promoter.

The nonsymbiotic hemoglobin may be barley hemoglobin.

The plant may be maize.

Preferably, the promoter is maize ubiquitin promoter.

According to a second aspect of the invention, there are provided plant cells transformed with any one of the expression systems described above.

According to a third aspect of the invention, there is provided a transgenic plant whose genome has been modified to contain the expression system described above.

According to a fourth aspect of the invention, there is provided a method of increasing tolerance to hypoxic conditions comprising:

providing an organism having increased cellular levels of an oxygen-binding protein having a low dissociation constant for oxygen; and

placing the organism under hypoxic conditions,

wherein the oxygen-binding protein acts to maintain cellular energy status during the hypoxic conditions by making oxygen available for cellular metabolism at low oxygen tension.

According to a fifth aspect of the invention, there is provided a method of lowering the level of fermentation products in an organism comprising:

providing an organism having increased cellular levels of an oxygen-binding protein having a low dissociation constant for oxygen; and

reducing the level of fermentation products in the cells of the organism by maintaining cell energy status such that fermentation is bypassed.

According to a sixth aspect of the invention, there is provided a method of maintaining cellular metabolism under hypoxic conditions comprising:

providing an organism having increased cellular levels of an oxygen-binding protein having a low dissociation constant for oxygen; and

placing the organism under hypoxic conditions,

wherein the oxygen-binding protein acts to maintain cellular metabolism status by providing oxygen for cellular metabolism.

According to a seventh aspect of the invention, there is provided a method of increasing oxygen uptake of an organism comprising:

providing an organism having increased cellular levels of an oxygen-binding protein having a low dissociation constant for oxygen; and

exposing the organism to an oxygen-containing environment,

wherein the increased cellular levels of the oxygen-binding protein results in increased oxygen uptake.

According to an eighth aspect of the invention, there is provided a method of improving the agronomic properties of a plant comprising:

providing a plant having increased cellular levels of an oxygen-binding protein having a low dissociation constant for oxygen; and

growing the plant.

The improved agronomic properties may include germination, seedling vigour, reduced cellular levels of fermentation products, increased oxygen uptake, and increased tolerance to hypoxic conditions.

One embodiment of the invention will now be described in conjunction with the accompanying figures in which:

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic diagram summarizing the structures of pAS1

and pAS2 respectively.

Figure 2 is the protein immunoblot analysis of hemoglobin expression in wild-type (BMS), HB^+ and HB^- maize cell lines with recombinant barley hemoglobin-specific antibody.

Figure 3 is a graph of the growth rate of wild-type (BMS), HB^+ and HB^- maize cell lines under normal atmospheric conditions.

Figure 4 is a bar graph comparison of oxygen uptake by maize wild-type (BMS), HB^+ and HB^- cells.

Figure 5 is a bar graph comparison of ATP levels in wild-type (BMS), HB^+ and HB^- maize cells grown under normal atmospheric conditions, after 12 hours of treatment with nitrogen, under normal atmospheric conditions following treatment with Antimycin A and after 12 hours of treatment with nitrogen following treatment with Antimycin A.

Figure 6 is a bar graph comparison of CO_2 evolution by maize cells cultured under a nitrogen atmosphere.

Figure 7 is a graph of alcohol dehydrogenase activity in maize cells cultured under a nitrogen atmosphere.

Figure 8 is a bar graph of oxygen uptake by maize cells under low oxygen atmosphere.

Figure 9 is a bar graph of oxygen uptake by maize cells under normal air conditions.

Figure 10 is a graph of cell culture growth following hypoxic treatment.

Figure 11 is a bar graph of the amount of hemoglobin in crude extracts

made from germinating barley seeds.

Table 1 is a summary of measurements of energy charge and total adenylates in maize cells before and after exposure to a nitrogen atmosphere for 12 hours.

DETAILED DESCRIPTION

Expression plasmids containing DNA encoding a nonsymbiotic hemoglobin, in this embodiment, barley hemoglobin, in both the sense and anti-sense orientation were constructed. The plasmids also included a strong constitutive promoter, in this embodiment, the maize ubiquitin promoter, and a selectable marker for selection of transformants, in this embodiment, a herbicide resistance gene (Bar), conferring resistance to glufosinate ammonium. The plasmids were transformed into cultured maize cells of the Black Mexican Sweet (BMS) variety, producing a cell line containing the sense plasmid (HB^+) and a cell line containing the antisense plasmid (HB^-).

When grown in an air environment, the HB^+ and HB^- cells did not differ significantly from wild-type BMS cells in terms of growth rate, oxygen consumption or cellular ATP levels. However, when grown under a nitrogen atmosphere, ATP levels in the HB^+ cells remained essentially the same as those observed unoer normal atmosphere conditions while ATP levels dropped significantly in wt and HB^- cells. Analysis of ATP levels in all three cell lines under a nitrogen atmosphere following treatment with Antimycin A (which blocks mitochondrial electron transport) indicated that the increase in ATP in HB^+ cells was not cytochrome-mediated. Furthermore,

measurements of CO_2 evolution and alcohol dehydrogenase activity in HB^+ cells suggested lower ethanolic fermentation rates in this cell line.

These data indicate that over-expression of nonsymbiotic hemoglobins helps maintain the energy status of cells grown at low oxygen tensions. This in turn has several possible applications, as cells capable of maintaining energy status at low oxygen tensions would have, for example, increased tolerance to a low oxygen atmosphere, improved germination rates and seedling vigour, be able to maintain cellular metabolism at low oxygen tension, reduce levels of fermentation products within the cells by lowering alcohol dehydrogenase activity, increase oxygen uptake under low oxygen tension and increase tolerance to hypoxic conditions such as, for example, ice encasement, flood and growth in compacted soil.

EXAMPLE I – CELL CULTURES

Black Mexican Sweet (BMS) (wild-type), HB^+ and HB^- maize cells were cultured in 250 ml flasks as cell suspensions in 50 ml of MS medium (Murashige and Skooge, 1962, *Physiol Plant* 15:473-497, incorporated herein by reference) macro and micro elements supplemented with thiamine 0.5 mg/litre, L-asparagine 150 mg/litre, 2, 4-D 2 mg/litre and sucrose 20 g/litre. Cultures were shaken at 150 rpm at 25°C. Cells were subcultured every 7 days. Nitrogen treatment was applied by replacing air in culture flasks with nitrogen and closing the flasks with rubber stoppers, otherwise culture flasks were closed with caps allowing for free exchange of air. Antimycin A was added as a 27 mM stock solution in 2-propanol to give a final concentration of 0.2 nM. Cell samples were collected by filtration. Cell samples used

for adenylate measurements were immediately frozen in liquid nitrogen and stored at –80°C until used.

EXAMPLE II – CONSTRUCTION OF VECTORS

SalI/NotI digested and end-filled barley hemoglobin cDNA was cloned into BamHI digested and end-filled pAHC17 plasmid (Christensen and Quail. 1996. *Transgenic Research* 5:213-218, incorporated herein by reference) in sense and antisense orientation to generate pAS1 (sense) and pAS2 (antisense) plasmids. An EcoRI digested end-filled, with synthetic HindIII linker 1.35 kb 35S promoter –bar gene- 35S terminator fragment from pDB1 (Becker et al. 1994. *Plant J* 5:299-307. incorporated herein by reference) into HindIII digested pAS1 and pAS2, as described below.

EXAMPLE III – TRANSFORMATION AND SELECTION

A silicon carbide fibres-mediated transformation system was used as described in Kaeppler et al., 1992, *Theor Appl Genet* 84:560-566, the disclosure of which is incorporated herein by reference, to transform BMS maize cells with pAS1 and pAS2 vectors. Resistant colonies were selected on culture medium solidified with 0.2% Phytagel™ (Sigma) and supplemented with glufosinate ammonium at a concentration of 5 mg/litre.

EXAMPLE IV.– PROTEIN IMMUNOBLOTS

SDS gel, protein transfer to nitrocellulose membrane and antibody

detection were performed according to standard Bio-Rad protocol (Bio-Rad bulletin 1721, incorporated herein by reference). Hemoglobin protein in transformec lines was detected by immunoblots, using a polyclonal antibody raised against barley recombinant hemoglobin. Protein concentration was calculated by densitometric comparison of immunoblots (in four repetitions) with a standard curve of known concentrations of recombinant Hemoglobin using a Sharp Diversity 1 PDI-325OE Scanner™.

EXAMPLE V – MEASUREMENTS

Culture growth was measured by sedimentation in 25 ml graduated pipettes. Adenylates were extracted in 1N perchloric acid from frozen cell samples at $-10^{o}C$ and ATP, ADP and AMP assayed spectrophotometrically by established protocols as described in Lowry and Passonneau, 1972, A Flexible System of Enzymatic Analysis, Academic Press: New York, which is incorporated herein by reference.

Alcohol dehydrogenase activity was measured in the ethanol – acetaldehyde direction in fresh cell extracts. Enzyme extraction and spectrophotometric measurements were performed as described in Hanson and Jacobsen, 1984, *Plant Physiol* **75**:566-572, which is incorporated herein by reference.

For measurements of CO_2 evolution from cell cultures, 1 ml gas samples were collected with an air tight syringe, from stoppered culture flasks, and analyzed by gas chromatography (Shimadzu GC-8AIT™).

Oxygen uptake was measured polarographically with an O_2 electrode

(Rank Brothers, Cambridge, UK) for 5 to 30 minutes. The incubation cell contained 2 ml of culture medium, 0.2 ml (sedimented cell volume) of cells. In some measurements, 0.2 mM Antimycin A was added, as described below.

EXAMPLE VI – EFFECT OF NONSYMBIOTIC HEMOGLOBIN ON CELL ENERGY STATUS

As noted above, cultured maize cells of the Black Mexican Sweet (BMS) variety were transformed with a barley hemoglobin gene to observe the effect of increasing or decreasing hemoglobin expression on cell metabolism. Specifically, transformation vectors, shown in Figure 1, were prepared containing the open reading frame of a barley hemoglobin cDNA in sense and antisense orientations, which were placed under the control of a strong constitutive promoter, in this embodiment, the maize ubiquitin (Ubi1) promoter. A herbicide resistance gene (Bar), conferring resistance to glufosinate ammonium, was cloned head to tail with the hemoglobin gene constructs to enable selection of transformed cell lines. Twenty-four independently transformed sense (pAS1) and thirty-eight anti-sense (pAS2) lines were obtained. Transformation was confirmed by Southern blot analysis and PCR. A sense line (HB^+) expressing hemoglobin at levels 10 fold higher than wild type (BMS) and an antisense line (HB^-) with 10 times lower expression of hemoglobin than BMS, as shown in Figure 2, were selected for further studies, as described below.

The three cell lines, grown in an air environment, did not differ significantly from one another with respect to culture growth rates, as shown in Figure 3, and consumption of oxygen, as shown in Figure 4. Furthermore, steady state ATP

levels wer essentially the same in the three types of cells, as shown in Figure 5. However, after incubation of the cells for a further 12 hours under an atmosphere of nitrogen gas, significant differences were observed in the ATP levels of the cell types. Specifically, the level of ATP was highest in HB^+ cells, being only marginally lower than under normal atmospheric conditions while ATP levels in wild type (BMS) cells were 27% lower than HB^+ cells and ATP levels in HB^- cells were 61% lower than HB^+ cells. Differences in energy charge and total adenylates were also observed in cells exposed to nitrogen atmospheres, as summarized in Table 1. As can be seen, energy charge was relatively the same in all three cell types under normal atmospheric conditions and in BMS and HB^+ cell lines after 12 hours of a nitrogen atmosphere. HB^- cells, on the other hand, were unable to maintain energy charge during the 12 hour exposure to a nitrogen atmosphere. Total adenylates remained the same in all three cell lines under atmospheric conditions and in HB^+ cells in a nitrogen atmosphere; however, in BMS and HB^- cells, the total adenylates declined by about 35 percent.

From this, it is evident that determining what part of the cell's metabolism contributes to this increased ability to maintain energy status in the presence of hemoglobin is critical to understanding the role of nonsymbiotic hemoglobin. To examine the possibility that hemoglobin might provide oxygen to generate ATP via cytochrome-mediated respiratory processes, Antimycin A (0.2 mM), which blocks mitochondrial electron transport in the span from cytochrome b to c and has been shown to induce hemoglobin expression in aleurone layers (Nie and Hill, 1997, *Plant Physiol* 114:835-840) was used. Antimycin A inhibited 80% of the oxygen uptake by maize cells within 30 minutes of treatment. After 12 hours exposure to

Antimycin A in an air environment, ATP levels in the three cell types were similar to those of untreated cells after 12 hours under a nitrogen atmosphere, as shown in Figure 5. However, upon placing Antimycin A-treated cells in a nitrogen atmosphere for 12 hours, the cell lines all showed decreases in ATP but, consistent with the previous experiments, the levels of ATP decreased in the order HB^+, BMS, and HB^-. This provides evidence that the increase in ATP brought about by the presence of hemoglobin was not the result of cytochrome-mediated mitochondrial respiration. It is also unlikely that the increased ATP is the result of oxyhemoglobin supporting mitochondrial alternative oxidase activity, which would increase substrate phosphorylation through glycolysis.

Furthermore, as shown in Figure 6, CO_2 evolution from hypoxic HB^+ cells was 20 to 30% lower than CO_2 levels evolved from BMS or HB^- cells, which would not be anticipated if the Krebs cycle was being maintained through alternative oxidase activity.

EXAMPLE VII – ALCOHOL DEHYDROGENASE LEVELS

An examination of alcohol dehydrogenase activity (ADH) in the cell lines showed that ADH increased in all three lines over the course of the experiments, but the ADH activity was significantly lower in the sense transformants (HB^+) than in antisense transformants (HB^-) or wild-type cells, as shown in Figure 7. Fluorescein diacetate staining (Heslop-Harrison et al., 1984, *Theor. Appl. Genet.* 67:367-375; incorporated herein by reference) showed no difference in the viability of the cell lines at the end of the incubation period. The reduced ADH activity, along with lower CO_2

evolution in HB^+ cells, likely reflects lower ethanolic fermentation rates, suggesting that a fermentative pathway may be the main s urce of carbon dioxide production in this system.

EXAMPLE VIII – OXYGEN UPTAKE

As discussed above, the presence of nonsymbiotic hemoglobin clearly affects the energy status of maize cells under hypoxia. Furthermore, differences between the HB^+, wild type and HB^- cells were observed only under the conditions of limited oxygen. To investigate the possibility that the observed differences may be due to the different abilities of the cell lines to utilize oxygen that is available in low concentrations, the oxygen uptake by the maize cells was measured under normal air conditions, shown in Figure 9, and in medium equilibrated with a mixture of 2% O_2 and 98% N_2, shown in Figure 8. Specifically, oxygen uptake was measured polarographically with an O_2 electrode. As can be seen, HB^+ cells were more efficient at oxygen uptake than the wild-type cells and much more efficient than the HB^- cells. Specifically, the oxygen uptake by the HB^+ cells from the medium equilibrated with 2% oxygen was 45% lower than that of all three cell lines under normal air conditions, as shown in Figures 8 and 9. Furthermore, wild-type BMS cells exhibited over 20% further decrease in the O_2 uptake rate, whereas the oxygen uptake by the oxygen uptake by the HB^- cells declined by a further 65%, as shown in Figures 8 and 9. These results clearly indicate that the rate of oxygen utilization by maize cells under low oxygen atmosphere depends on the presence of the non-symbiotic hemoglobin.

EXAMPLE IX – CELL GROWTH AFTER EXPOSURE TO HYPOXIC STRESS

The ability of the cell cultures to continue growth after exposure to hypoxic stress was also tested. Maize cell cultures were placed under the atmosphere of nitrogen for 12 and 24 hours, then cells were harvested, transferred to a fresh medium and their growth was monitored by sedimented cell volume measurements, as shown in Figure 10. Upon placement under the N_2 atmosphere, the cell growth of all three cell lines ceased, but resumed after transfer to the fresh medium and normal atmospheric conditions. However, while the HB^+ cell cultures resumed growth almost immediately after the transfer to normal air conditions, the HB^- cells showed a 36 hour lag period before commencement of intensive growth. Furthermore, the growth of the wild-type cultures, during the first 36 hours after the transfer to normal conditions, was slower than that of HB^+ cells, as shown in Figure 10. It is of note that after the initial 36 hour period, the growth rates of the three cell lines were almost identical. The differences in cell volume at each time point were most likely a result of the growth activity during this initial period. The culture re-growth after the 24 hour hypoxic exposure was the same for all three cell lines, as after the 12 hour treatment. The observed differences may be explained by different levels of cell survival under stress, and, depending on the cell line, the same cell volume could contain different numbers of growing cells. On the other hand, the steep growth of the HB^- and the wild-type BMS cultures after a lag period, shown in Figure 10, suggest a longer stress recovery period rather than cell death.

EXAMPLE X – HEMOGLOBIN EXPRESSION IN GERMINATING BARLEY

Polyclonal antibodies to purified recombinant barley hemoglobin were raised in rabbits and used to investigate the expression of hemoglobin in monocotyledonous plants. Specifically, hemoglobin was shown to be expressed in whole seeds, as shown in Figure 11, embryo-less half seeds and excised embryos during germination. The fact that hemoglobin was expressed in both embryo-less half seeds and excised embryos indicates that the gene is independently responsive to signals in both tissues and suggests that both the aleurone layer and the embryo may experience oxygen deficiencies during the imbibition process. In the excised embryo, hemoglobin was induced between 4 and 6 hours after imbibition. Since germination and the early stages of seedling growth are known to be periods of high metabolic demand (Bewley and Black, 1990, *Prog Nucleic Acid Res Mol Biol*, 38:165-193, incorporated herein by reference), this data is consistent with the proposed concept that a demand on energy charge or ATP requirement is primarily responsible for hemoglobin induction (Nie and Hill, 1997, *Plant Physiol* 114:835-840, incorporated herein by reference). Major changes in ATP content of the embryos did occur within one hour after imbibition, which is consistent with previous reports. Protein hydration, protein synthesis and nucleotide synthesis are among the first events of germination. These early events, which consume large amounts of ATP, may well be a factor in the observed induction of hemoglobin synthesis at 4 to 6 hours after imbibition. However, induction occurs well before the major increase in α-amylase secretion, a period of high metabolic demand, and so the relationship between hemoglobin synthesis and energy availability needs further clarification.

In half seeds, there is an apparent induction of hemoglobin during

imbibition, without the use of gibberellic acid to stimulate the synthesis of hydrolytic enzymes. Furthermore, isolated aleurone layers do not show appreciable amounts of hemoglobin unless induced by anoxia using a nitrogen environment (Nie and Hill, 1997). The aleurones in these half-seeds may well be experiencing anoxia due to entrapment in the endosperm and seed coat.

Thus, to summarize, very little or no hemoglobin expression was observed in dry barley seeds but germination resulted in the expression of hemoglobin which peaked at 2-3 days after imbibition, as shown in Figure 11. Furthermore, hemoglobin expression was also observed in maize, wheat, wild oat and *Echinochloa crus galli* seeds during germination. Dissection of tissues from the barley seedlings showed that most of the hemoglobin was expressed in the root and seed coat (aleurone layer), with very little in the coleoptile. Imbibition of half seeds or excised embryos resulted in the expression of hemoglobin. ATP measurements of barley embryos showed that ATP levels quickly increase after imbibition. α-amylase activity was also determined in the embryos to correlate hemoglobin expression with a well-characterized germination response. The results demonstrate that hemoglobin expression is a normal consequence of germination.

EXAMPLE XI– DISCUSSION

Higher plant hemoglobins are cytoplasmic proteins (Wittenberg and Wittenberg, 1990). With this in mind, transformation constructs were designed for cytoplasmic expression of hemoglobin. Barley hemoglobin cDNA hybridizes to only one locus in barley and maize genomes (Taylor et al, *Plant Mol Biol* 24:853-862,

incorporated herein by reference) and, therefore, sense and antisense expression of this cDNA would not be expected to affect the expression of any other genes. It is of note that the polyclonal anti-hemoglobin antibody used was raised and titrated against recombinant barley hemoglobin. Furthermore, it is clear that there is over and under expression of hemoglobin in the transgenic cells.

The lack of effect of hemoglobin on cell growth and oxygen uptake under normal air conditions likely reflects the fact that barley (Taylor et al, 1994) and maize hemoglobin genes are induced under conditions of limited oxygen availability, resulting in the protein having little effect when oxygen supplies are not impaired. The results, however, show clearly that the energy status of maize cells when oxygen is limiting is affected by the ability of the cells to produce hemoglobin. Total adenylates and ATP levels are maintained during the period of exposure to limiting oxygen when hemoglobin is constitutively expressed in the cells. Alternatively, when hemoglobin expression is suppressed by constitutive expression of antisense barley hemoglobin message, the cells are unable to maintain their energy status during oxygen limitation. In wild-type (BMS) cells, it would appear that the induction of native maize hemoglobin was sufficient to maintain the energy charge, but not the total adenylate pool. This is consistent with the observation that a decline in the adenylate pool has been noted during hypoxia in maize root tips (Saint-Ges et al, 1991, *Eur J Biochem* **200**:477-482). Under limiting oxygen, plant cells turn their metabolism towards fermentation in order to oxidize NADH necessary to maintain glycolytic substrate phosphorylation. Lower alcohol dehydrogenase activity in HB^+ cells suggests that hemoglobin provides an alternative to potentially harmful fermentation. Specifically,

carbon dioxide is produced by the HB^+ cells in lower amounts than by HB^- and wild-type maize cells, reflecting lower ADH activity and suggesting that the ethanolic fermentation is the only source of CO_2. The dissociation constant of barley oxyhemoglobin is about 3 nM (Duff et al, 1997, incorporated herein by reference), indicating that oxyhemoglobin, acting alone, would be ineffective in providing oxygen to maintain mitochondrial respiratory processes. This is confirmed by the observation that Antimycin A has no effect on the ability of hemoglobin-containing cells in maintaining their energy status under low oxygen tensions. The results discussed above suggest that hemoglobin maintains energy status of the cell by means different from mitochondrial oxidative phosphorylation , probably by facilitating glycolysis to generate ATP through substrate level phosphorylation.

It is of note that hemoglobins of barley (Taylor et al, 1994) and maize as well as *Arabidopsis* AHB1 (Trevaskis et al, 1997) are hypoxia inducible. Furthermore, it has been demonstrated that, in barley hemoglobin, this is not due to a lack of oxygen per se, but in response to insufficient mitochondrial ATP synthesis. In addition, nonsymbiotic hemoglobins are expressed in metabolically active tissues such as roots (Taylor et al, 1994; Arredondo-Peter et al, 1997; Trevaskis, 1997), aleurone (Taylor et al, 1994), vascular tissues of leaves, stems and seedling cotyledons (Andersson et al, 1996, *Proc Natl Acad Sci* 93:5682-5687). Taken together, these data support a hypothesis that nonsymbiotic hemoglobins utilize available oxygen in cells exposed to low oxygen tensions or other concitions that reduce cellular ATP levels to maintain the cell's energy status. The very low dissociation constant of barley oxyhemoglobin makes it an ideal candidate for

sequestering oxygen in low oxygen environments. Interaction with another compound, perhaps a flavoprotein, could create a complex capable of oxidizing NADH, in a manner analogous to Hmp protein of *E. coli* (Poole et al, 1996, *Microbiology (Reading)* **142**:1141-1148). This would provide an efficient means of oxidatively regenerating NAD to support glycolysis, bypassing the fermentative route to ethanol.

The effects of expression of sense and antisense hemoglobin on energy charge are reminiscent of hypoxic acclimation of plant tissues, for example, maize root tips, which develop a tolerance to short term anoxia if they have been acclimated by exposure to hypoxic conditions (Bennoun, 1982, *Proc Natl Aced Sci* **79**:4352-4356; Johnson et al, 1989, *Plant Physiol* **91**:837-841). Specifically, acclimation is accompanied by increased energy charge (Hole et al, 1992, *Plant Physiol* **99**:213-218) resulting from a sustained glycolytic rate compared to non-acclimated root tips (Xia and Saglio, 1992, *Plant Physiol* **100**:40-46; Xia and Roberts, 1996, *Plant Physiol* **111**:227-233). Similarly, winter cereals show increased survival to hypoxia caused by ice encasement if they have been acclimated by exposure to hypoxic conditions (Andrews and Pomeroy, 1983, *Can J Bot* **61**:142-147). Acclimated plants maintain higher levels of adenylates and ATP during ice encasement, as a result of accelerated rates of glycolysis, than non-acclimated plants (Andrews and Pomeroy, 1989, *Plant Physiol* **91**:1063-1068). Maximum induction of barley hemoglobin message occurs within 12 hours exposure to hypoxic conditions (Taylor et al, 1994), which is well within the time interval used for acclimation in the above examples. Furthermore, it has been shown that the expression of hemoglobin is not directly influenced by oxygen usage or availability but it is influenced by the availability of ATP in the tissue

(Nie and Hill, 1997). This suggests that the increased survival of plants to anoxia as a result of hypoxic acclimation is a consequence of hemoglobin gene induction due to declining ATP levels during acclimation

From an evolutionary standpoint, it has been suggested that nonsymbiotic hemoglobins represent one of the more ancient forms of plant hemoglobins (Andersson et al, 1996). Evidence presented here adds credence to this idea. Since early life on earth existed in oxygen-poor environments, the presence of a hemoglobin capable of utilizing oxygen at low oxygen tensions would have provided an evolutionary advantage to an organism. Oxygen produced during photosynthesis and retained as oxyhemoglobin would provide a source of oxygen to oxidize NADH, maintaining a high glycolytic flux during darkness to provide ATP for cell growth and development.

The high oxygen avidity of hemoglobin (Arredondo-Peter et al, 1997; Duff et al, 1997; Trevaskis et al, 1997, all incorporated herein by reference) argues against hemoglobin functioning to facilitate diffusion of oxygen. Because the hemoglobin will be induced intracellularly in a highly reductive environment with low energy charge it is possible that hemoglobin functions as an electron transport protein similar to cytochrome c. Further work is now being carried out to more closely examine the potential effect of oxygen limitation and hemoglobin expression during germination.

The function of this enigmatic protein is still far from certain. We have observed hemoglobin gene expression (or increases in hemoglobin expression) unequivocally in at least 4 cases: (1) in intact whole seeds during germination; (2) in

excised embryos and embryo-less half seeds imbibed in water; (3) in aleurone layers which have be n stressed by a low oxygen environment or respiratory inhibitors (Nie and Hill, 1997); and (4) in barley roots after flooding (Taylor et al, 1994). In every situation, it is likely that the ATP requirement of the cell exceeds the ATP supply either because of low oxygen supply (such as is the case of the flooded plants or stressed seed tissue) or due to high metabolic rates (such as likely to be the case during germination). Hemoglobin expression seems to be both a normal event during seed germination as well as an adaptation of plants to low oxygen environments.

Since various modifications can be made in our invention as herein above described, and many apparently widely different embodiments of same made within the spirit and scope of the claims without department from such spirit and scope, it is intended that all matter contained in the accompanying specification shall be interpreted as illustrative only and not in a limiting sense.

CLAIMS

1. A recombinant expression system capable, when transformed into a plant, of expressing a gene encoding a nonsymbiotic hemoglobin, which system comprises a nucleotide sequence encoding said nonsymbiotic hemoglobin operably linked to control sequences effective in said plant.

2. The system according to claim 1 wherein the control sequences include a strong constitutive promoter.

3. The system according to claim 1 wherein the nonsymbiotic hemoglobin is barley hemoglobin.

4. The system according to claim 3 wherein the plant is maize.

5. The system according to claim 4 wherein the promoter is maize ubiquitin promoter.

6. Plant cells transformed with the expression system according to any one of claims 1 to 5.

7. A transgenic plant whose genome has been modified to contain the expression system according to any one of claims 1 to 5.

8. A method of increasing tolerance to hypoxic conditions comprising:

providing an organism having increased cellular levels of an oxygen-binding protein having a low dissociation constant for oxygen; and

placing the organism under hypoxic conditions,

wherein the oxygen-binding protein acts to maintain cellular energy status during the hypoxic conditions by making oxygen available for cellular

metabolism at low oxygen tension.

9. A method of lowering the level of fermentation products in an organism comprising:

providing an organism having increased cellular levels of an oxygen-binding protein having a low dissociation constant for oxygen; and

reducing the level of fermentation products in the cells of the organism by maintaining cell energy status such that fermentation is bypassed.

10. A method of maintaining cellular metabolism under hypoxic conditions comprising:

providing an organism having increased cellular levels of an oxygen-binding protein having a low dissociation constant for oxygen; and

placing the organism under hypoxic conditions,

wherein the oxygen-binding protein acts to maintain cellular metabolism status by providing oxygen for cellular metabolism.

11. A method of increasing oxygen uptake of an organism comprising:

providing an organism having increased cellular levels of an oxygen-binding protein having a low dissociation constant for oxygen; and

exposing the organism to an oxygen-containing environment,

wherein the increased cellular levels of the oxygen-binding protein results in increased oxygen uptake.

12. A method of improving the agronomic properties of a plant comprising:

providing a plant having increased cellular levels of an oxygen-binding protein having a low dissociation constant for oxygen; and

growing the plant.

13. The method according to claim 12 wherein the improved agronomic properties include germination.

14. The method according to claim 12 wherein the improved agronomic properties include seedling vigour.

15. The method according to claim 12 wherein the improved agronomic properties include reduced cellular levels of fermentation products.

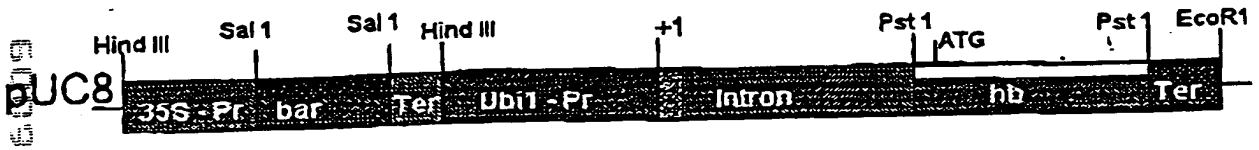
16. The method according to claim 12 wherein the improved agronomic properties include increased oxygen uptake.

17. The method according to claim 12 wherein the improved agronomic properties include increased tolerance to hypoxic conditions.

ABSTRACT

Nonsymbiotic hemoglobins are broadly present across the plant kingdom, however, the function of these proteins is unknown. Cultured maize cells have been transformed to constitutively express a barley hemoglobin gene in either the sense (HB^+) or antisense (HB^-) orientation. Hemoglobin protein in the transformed cell lines was correspondingly higher or lower than in wild type cells under normal atmospheric conditions. Limiting oxygen availability, by placing the cells in a nitrogen atmosphere for 12 hours, had little effect on the energy status of cells constitutively expressing hemoglobin, but had a pronounced effect on both wild type and HB^- cells, where ATP levels declined by 27% and 61% respectively. Total adenylates in these cells were approximately 35% lower than in HB^+ cells. Energy charge was relatively unaffected by the treatment in HB^+ and wild type cells, but was reduced from 0.91 to 0.73 in HB^- cells suggesting that the latter were incapable of maintaining their energy status under the low oxygen regime. Treatment of the cells grown in air atmosphere with antimycin A gave essentially the same results. It is suggested that nonsymbiotic hemoglobins act in plants to maintain the energy status of cells in low oxygen environments and that they accomplish this effect by promoting glycolytic flux through NADH oxidation, resulting in increased substrate level phosphorylation. Hypoxic acclimation of plants is an example of this effect in nature. Nonsymbiotic hemoglobins are likely ancestors of an early form of hemoglobin that sequestered oxygen in low oxygen environments, providing a source of oxygen to oxidize NADH to provide ATP for cell growth and development.

A. pAS1 (Sense)



B. pAS2 (Anti-sense)

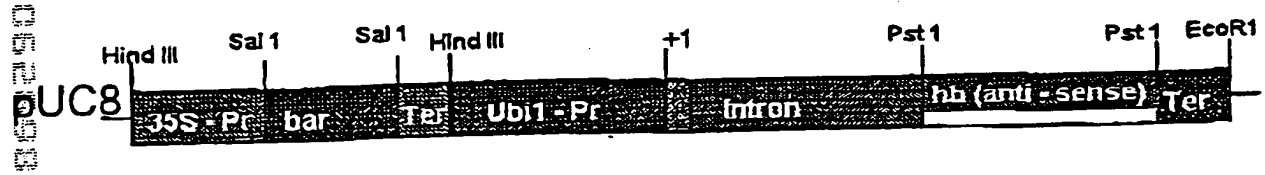
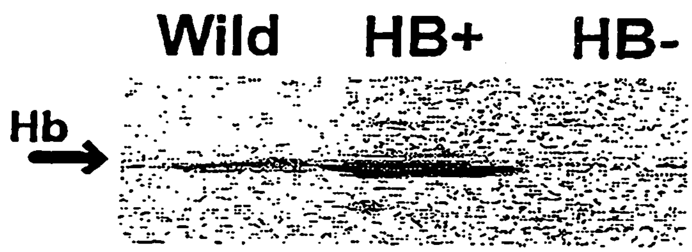


FIG. 1



	Wild	HB+	HB-
Fraction of total soluble protein [%]	0.12	1.18	0.016

FIG. 2

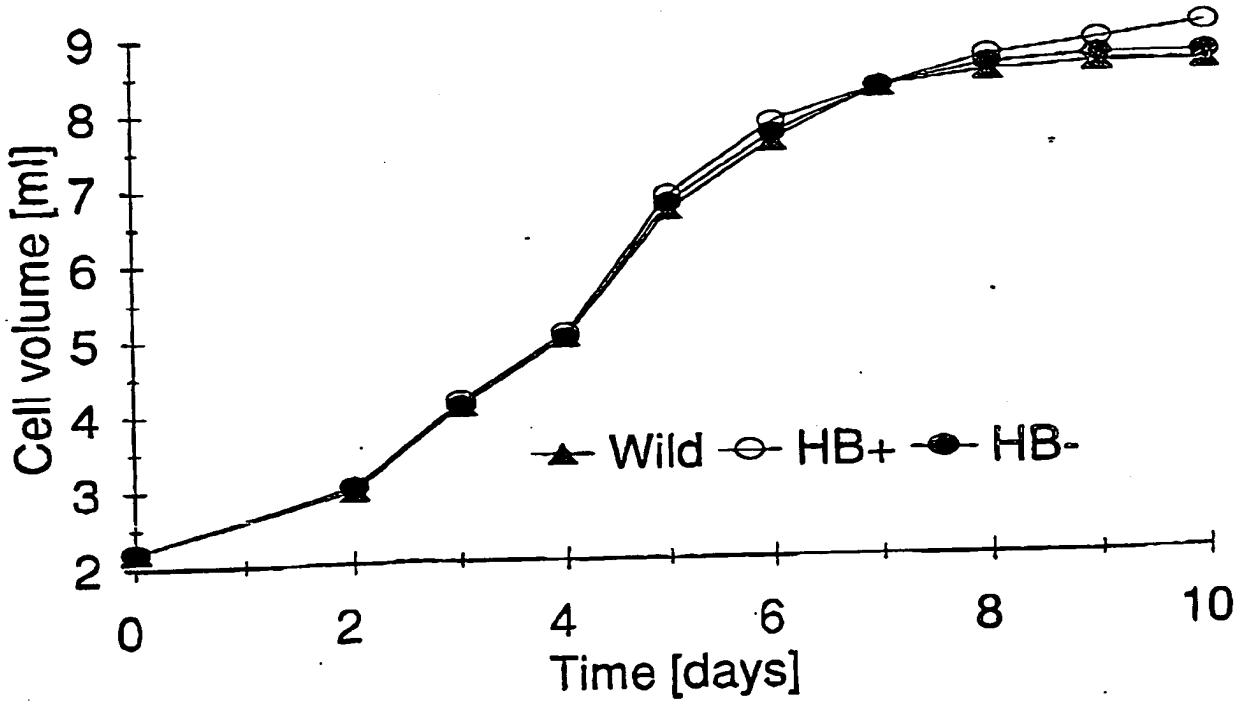


FIG. 3

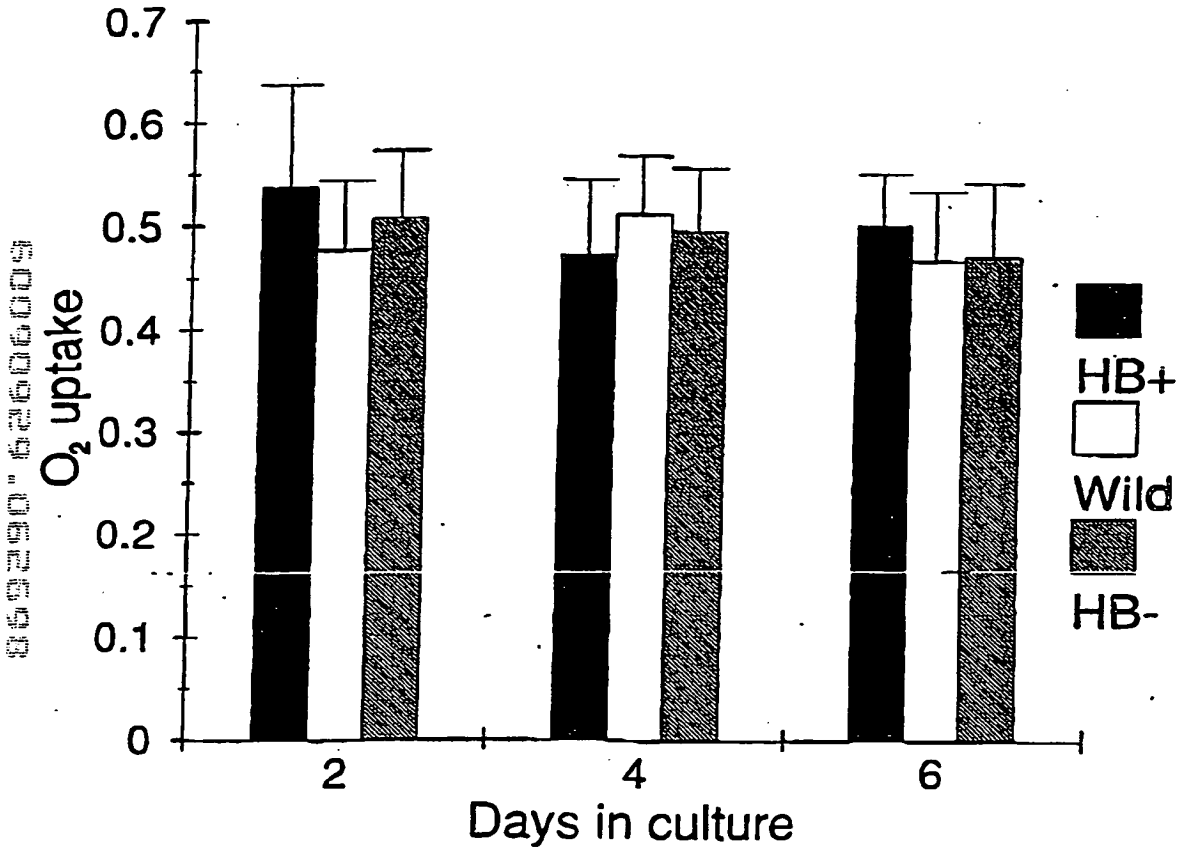


Fig. 4

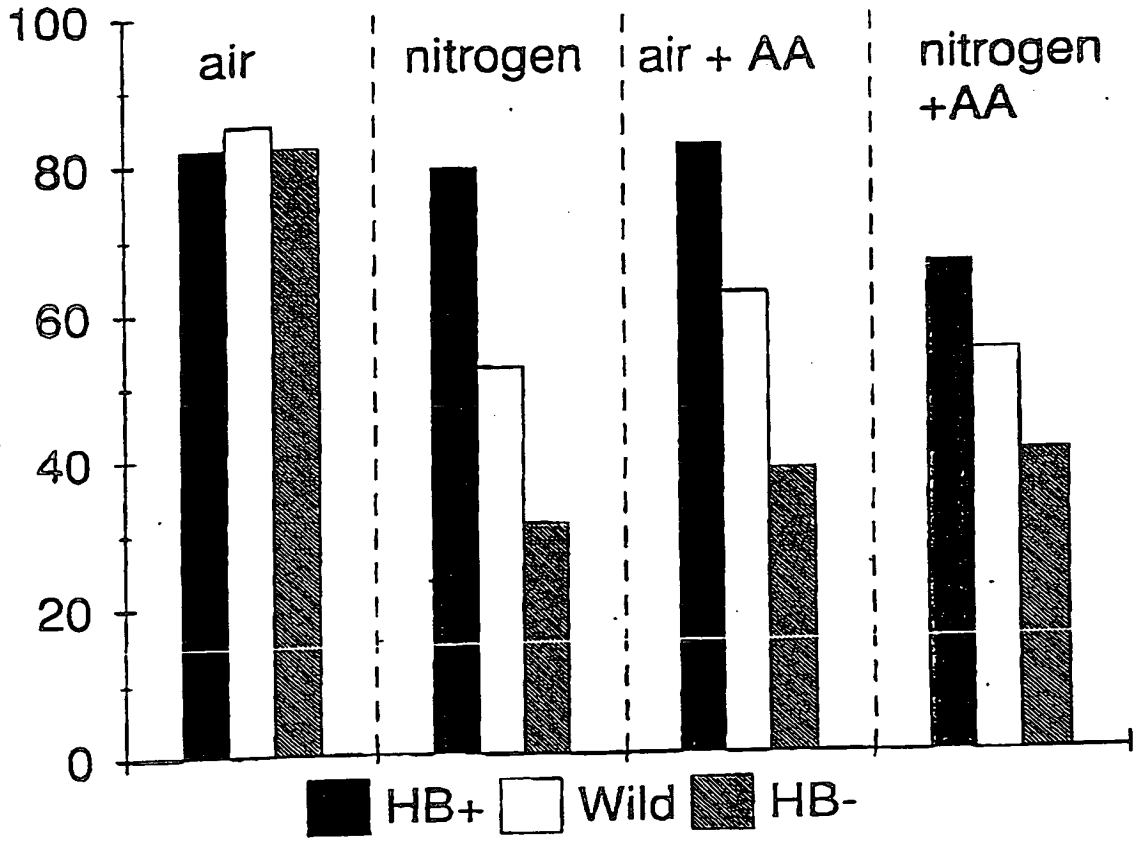

100
80
60
40
20
0
ATP [nmol/gfw]
air
nitrogen
air + AA
nitrogen
+AA
HB+
Wild
HB-

FIG. 5

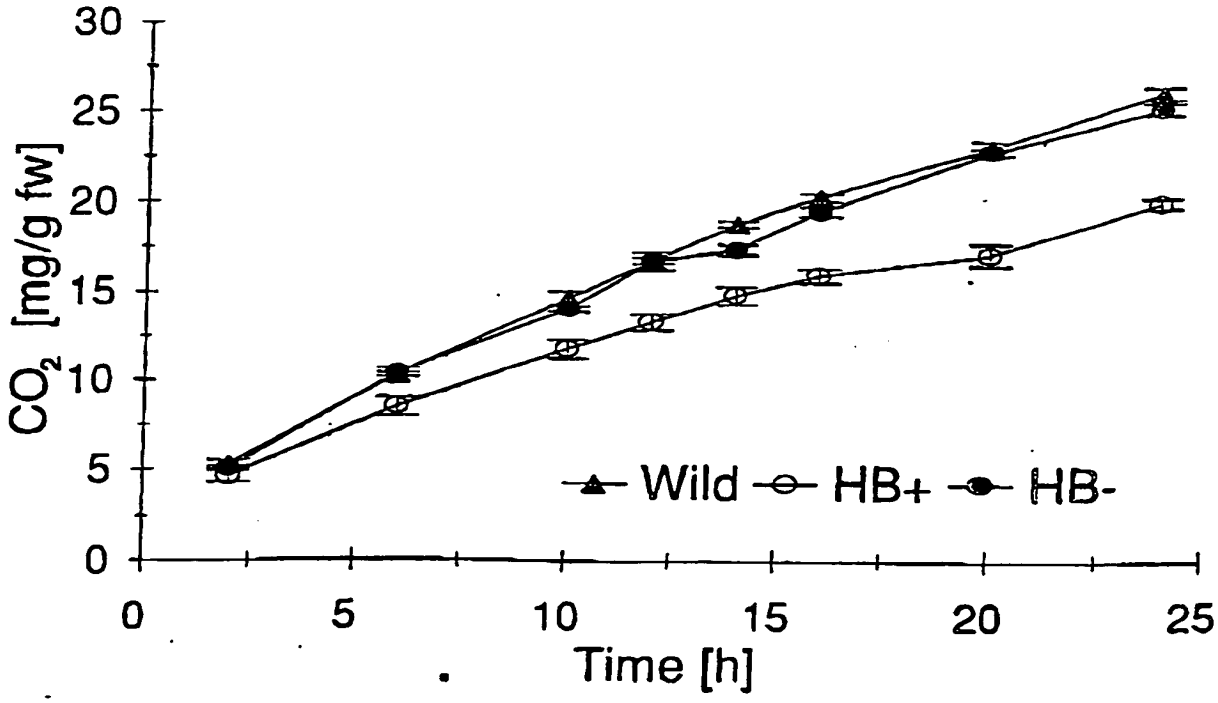


FIG. 6

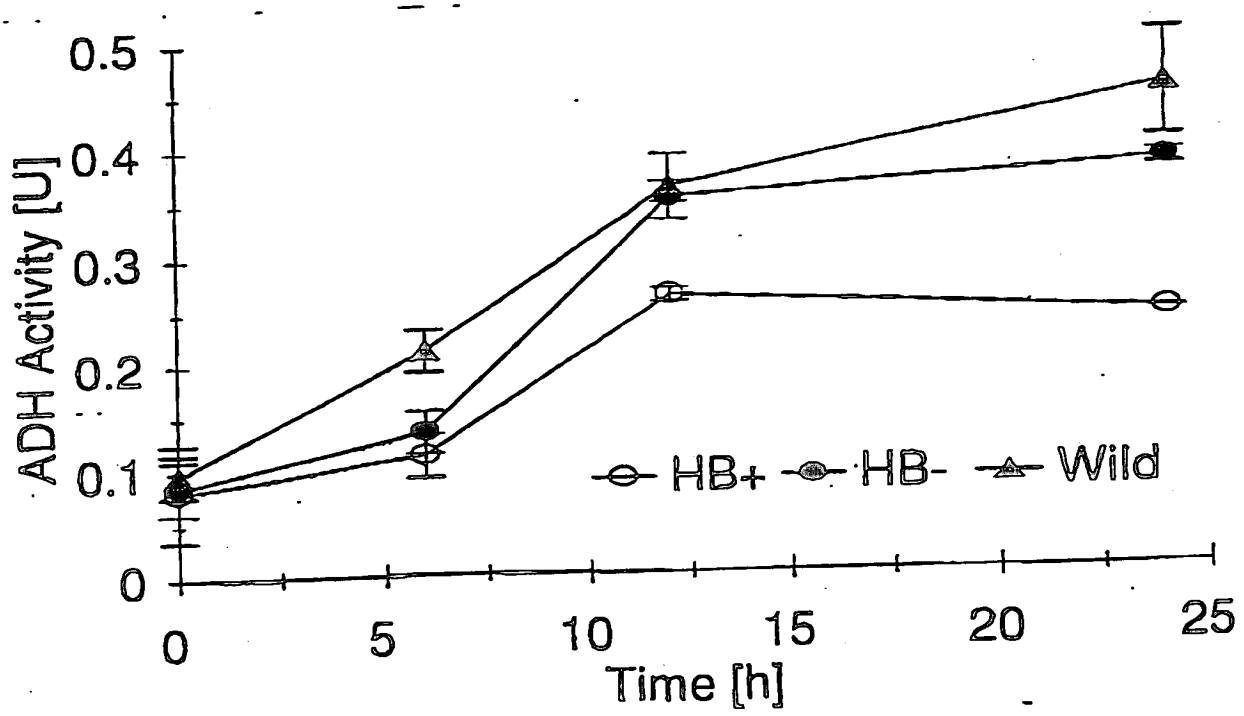


FIG. 7

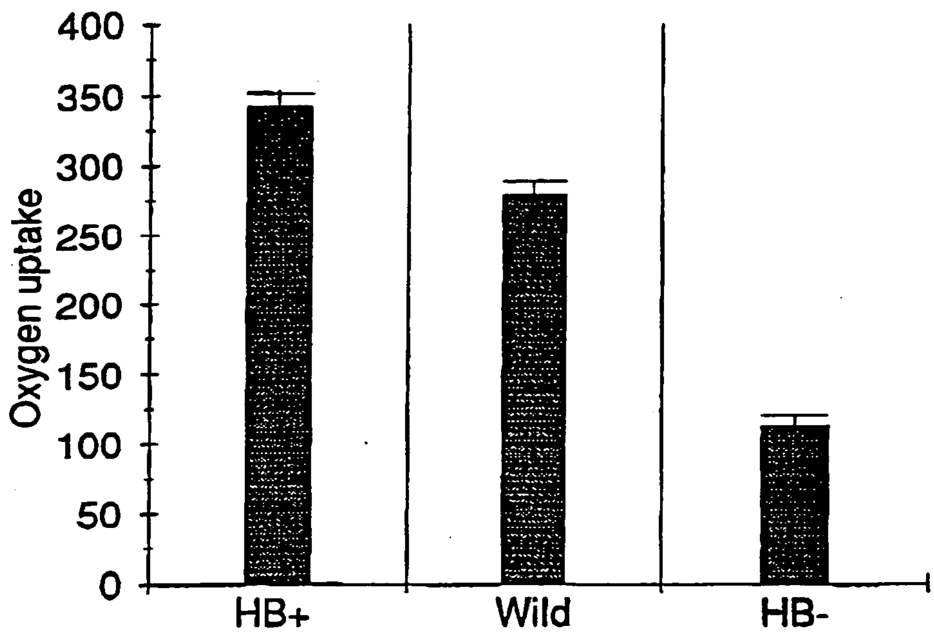


FIG. 8

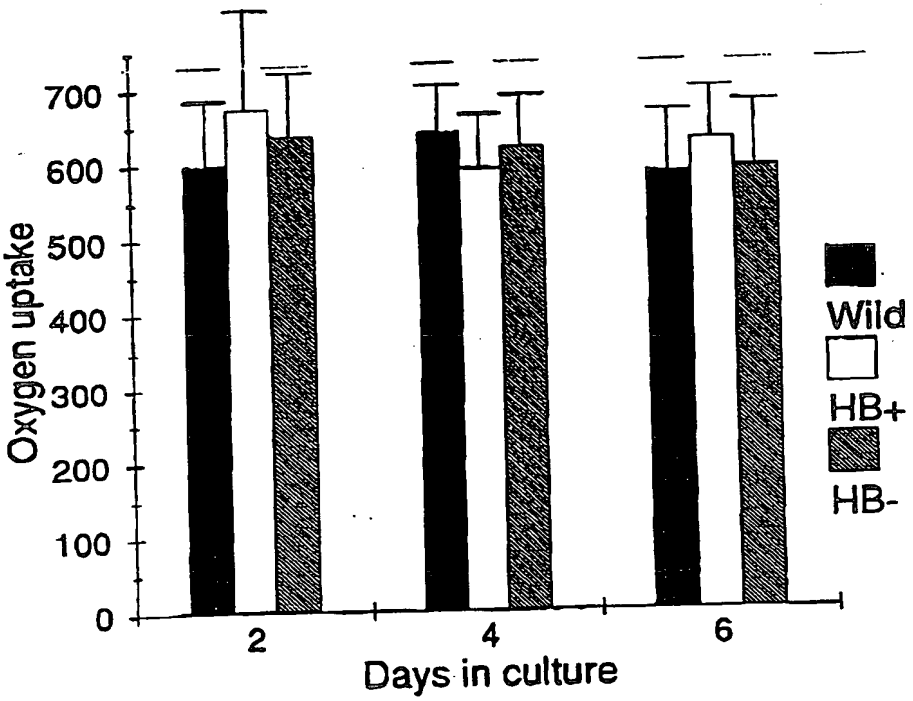
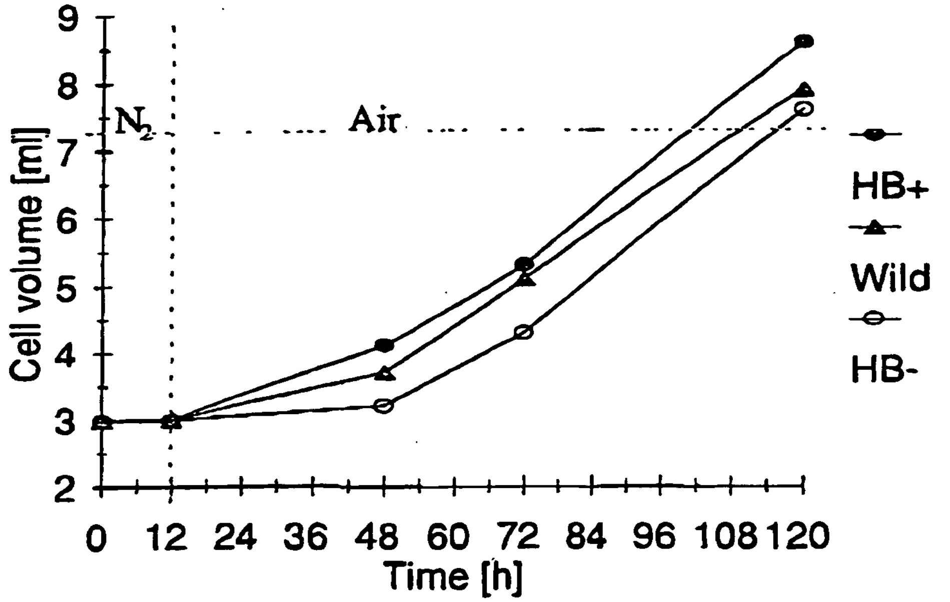

Oxygen uptake
700
600
500
400
300
200
100
0
2
4
6
Days in culture
Wild
HB+
HB-

FIG. 9



9
8
7
6
5
4
3
2
Cell volume [ml]
N2
Air
0
12
24
36
48
60
72
84
96
108
120
Time [h]
HB+
Wild
HB-

FIG. 10

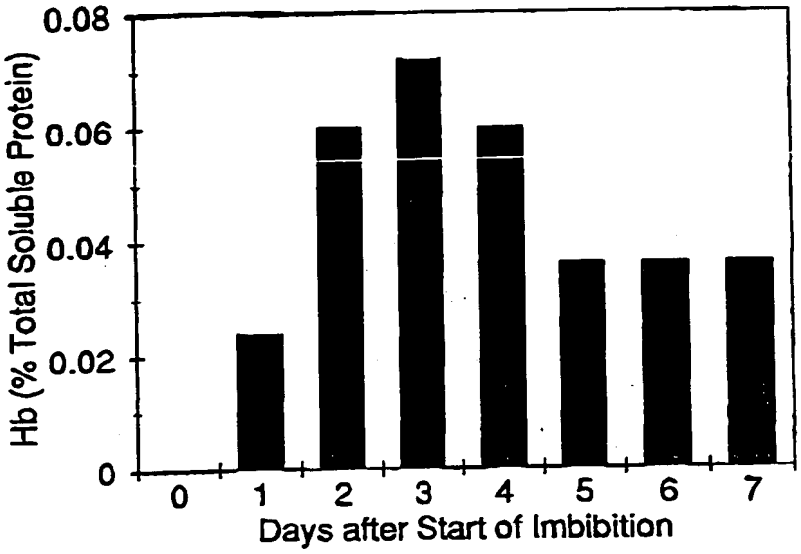

0.08
0.06
0.04
0.02
0
Hb (% Total Soluble Protein)
0
1
2
3
4
5
6
7
Days after Start of Imbibition

FIG. 11

Table 1. Energy charge and total adenylates in maize cells before and after exposure to a nitrogen atmosphere for 12 hours. Results are expressed as nmol per g fresh weight. Maximum SE (n = 3) was 5%.

Cell line	Energy Charge		Total Adenylates (nmol per g fresh weight)	
	Air	Nitrogen	Air	Nitrogen
HB^+	0.93	0.93	96	92
Wild	0.94	0.93	94	6
HB^-	0.91	0.73	99	59